NON-RESPONSE TO ANTIVIRAL THERAPY IS ASSOCIATED WITH OBESITY AND INCREASED HEPATIC EXPRESSION OF SOCS-3 IN PATIENTS WITH CHRONIC HEPATITIS C, VIRAL GENOTYPE 1.

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Key words: insulin resistance, TNF-α, PEPCK, suppressor of cytokine signalling.

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Abbreviations: IR, insulin resistance; SOCS, suppressor of cytokine signalling; HCV, hepatitis C virus; IFN, interferon-α; IFN-R1, interferon receptor 1; PEPCK, phosphoenolpyruvate carboxykinase; NR, non-response to antiviral treatment; RES, response to antiviral treatment; SVR, sustained virological response; BMI, body mass index; RT-PCR, real-time polymerase chain reaction; TNF-α, tumor necrosis factor-alpha.

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Abstract

Introduction: Interferon-α (IFN) -activated cellular signalling is negatively regulated by inhibitory factors including the suppressor of cytokine signalling (SOCS) family. The effects of host factors such as obesity on the hepatic expression of these inhibitory factors in subjects with chronic HCV are unknown.

Objectives: To assess the independent effects of obesity, insulin resistance (IR) and steatosis on response to IFN therapy and to determine the hepatic expression of factors inhibiting IFN signalling in obese and non-obese subjects with chronic HCV.

Methods: 145 subjects were analysed to determine host factors associated with non-response to antiviral therapy. Treatment comprised IFN or peginterferon-alpha either alone or in combination with ribavirin. In a separate cohort of 73 patients, real time (RT)-PCR was performed to analyse hepatic mRNA expression. Immunohistochemistry for SOCS-3 was performed on liver biopsy samples from 38 patients with viral genotype 1 who had received antiviral treatment.

Results: Non-response (NR) to treatment occurred in 55% of patients with HCV genotypes 1 or 4 and 22% with genotypes 2 or 3. Factors independently associated with NR were viral genotype 1/4 (p<0.001), cirrhosis on pre-treatment biopsy (p=0.025) and BMI ≥ 30kg/m² (p=0.010). Obese subjects with viral genotype 1 had increased hepatic mRNA expression of PEPCK (p=0.01) and SOCS-3 (p=0.047), in comparison with lean subjects. Following multivariate analysis, SOCS-3 mRNA expression remained independently associated with obesity (p=0.023). SOCS-3 immunoreactivity was significantly increased in obesity (p=0.013) and in NR compared with responders (p=0.014).

Conclusions: In patients with chronic HCV viral genotype 1, increased expression of factors that inhibit interferon signalling may be one mechanism by which obesity reduces the biologic response to IFN.
**Introduction**

Despite an improvement in the efficacy of antiviral treatment in recent years, approximately 50% of patients infected with hepatitis C virus (HCV) genotype 1 and 20% of those with HCV genotype 3 fail to achieve sustained viral clearance.[1] Along with viral genotype and load [2], various host genetic and biological factors have a role in the resistance to interferon-alpha (IFN) therapy. These host factors include gender, age, ethnicity and genetic variation in human leucocyte antigens and cytokine production.[reviewed in 3] In addition, patients with advanced fibrosis have a decreased response to antiviral treatment.[4]

In order to increase the number of patients achieving a sustained virologic response (SVR), there is a need to identify modifiable risk factors that impact on treatment efficacy. As a result, there is increasing interest in the role of obesity and hepatic steatosis in this setting. Steatosis may adversely affect the response to antiviral therapy.[5][6] In patients with non-genotype 3 infection, the presence of steatosis was a predictor of failed treatment [7][8] and those with less steatosis were more likely to achieve SVR.[6] However it is unlikely that steatosis intrinsically impairs antiviral efficacy since steatosis associated with viral genotype 3 does not appear to adversely affect the response to treatment.[6] Other investigators found that an elevated body mass index (BMI) in the obese range of greater than 30 kg/m^2, rather than steatosis per se, was associated with therapeutic non-response.[9]

Obesity and steatosis are clearly inter-related and it remains unclear whether their reported effects are truly independent.[10] A number of studies have found that patients with higher body weight have reduced response rates following antiviral therapy.[11][12][13][14] In the absence of weight-based dosing, treatment failure in obese patients may be due to inadequate drug doses leading to lower serum levels of IFN.[15] Other mechanisms by which obesity may impair antiviral response such as by reducing the biologic response to IFN are not well understood. Interaction of IFN with a cell surface receptor leads to a series of intracellular reactions that result in transcriptional induction of several antiviral and immunoregulatory genes.[3] This IFN-activated signalling is negatively regulated by a number of inhibitory factors including the suppressor of cytokine signalling (SOCS) family.[3][16][17][18] Several studies have found that patients with high tumor necrosis factor-alpha (TNF-α) levels have a poor response to IFN therapy [19][20][21] and this may occur via induction of SOCS proteins [22] that interfere with the interaction between the IFN receptor and signalling proteins.[16] Obesity and steatosis are known to be associated with elevated levels of TNF-α [23][24] and more recent studies have shown increased hepatic expression of SOCS proteins in insulin-resistant states.[25][26]

The aims of this study were to evaluate the independent effects of obesity, hepatic steatosis and obesity-related metabolic factors on the response to antiviral therapy in patients with chronic HCV infection. In addition, we examined the hepatic expression of the IFN receptor 1 (IFN-R1) and factors that may inhibit IFN signalling (TNF-α, SOCS-3) and phosphoenolpyruvate carboxykinase (PEPCK) as a marker of hepatic insulin sensitivity in obese and non-obese subjects with chronic HCV.

**Materials and Methods**

**Study populations**

This study was a retrospective review of patients with chronic HCV seen at a single
centre between 1995 and 2004. Subjects were included in the current study if they fulfilled the following criteria: a) chronic HCV with circulating HCV RNA (detected by Amplicor HCV Monitor assay, Roche, New Jersey, USA) and abnormal serum aminotransferase levels for at least 6 months b) liver biopsy consistent with chronic hepatitis c) compensated liver disease and d) written informed consent for inclusion in the study. Patients with other forms of chronic liver disease or antibodies to human immunodeficiency virus were not considered for the analysis. The study protocol was approved by both the Princess Alexandra Hospital Research Ethics Committee and the University of Queensland Research Ethics Committee.

145 subjects received antiviral therapy. Treatment comprised IFN or peginterferon-alpha (PEG-IFN) either alone or in combination with ribavirin. Since the purpose of the study was to determine the effect of host factors on treatment outcome, only subjects who completed 80% of the intended doses of antiviral therapy were evaluated. In an additional group of 73 untreated patients with chronic HCV, liver tissue (2-3mm) was immediately frozen in liquid nitrogen at the time of biopsy, and stored at −80°C until the extraction of RNA was performed.

Details about weight, height and average alcohol intake (g/day) were obtained from all patients at the time of treatment and/or liver biopsy. Information regarding average alcohol intake (g/day) prior to the last 6 months was also obtained. Subjects receiving antiviral therapy were required to consume < 70 g ethanol per week for ≥ 6 months prior to treatment and were abstinent during treatment.

Gender, ethnicity and age at treatment were also recorded. On the basis of BMI and ethnicity, subjects were classified as lean (Caucasian < 25 kg/m², Asian < 22.5 kg/m²), overweight (Caucasian 25-29.9 kg/m², Asian 23-24.9 kg/m²) or obese (Caucasian ≥ 30 kg/m², Asian ≥ 25 kg/m²).

**Histopathological examination**
Liver biopsy sections were analysed by an experienced hepatopathologist (AC) who was blinded to the laboratory parameters and clinical data. The degree of inflammation was graded according to the method of Ishak [27] and fibrosis was staged according to the method of Scheuer.[28] Steatosis was graded as follows: 0 (<5% hepatocytes affected); 1 (mild, 5-29% of hepatocytes affected); 2 (moderate, 30-70% of hepatocytes affected); or 3 (severe, >70% of hepatocytes affected).

**Laboratory data**
Viral genotyping was performed using the Inno-Lipa HCV II assay (Innogenetics, Zwijnaarde, Belgium). Circulating HCV RNA was detected by polymerase chain reaction (PCR) using the Amplicor HCV assay (Roche, New Jersey, USA). SVR was defined as undetectable HCV RNA at the end of 24 weeks follow-up after completion of treatment. Patients were responders (RES) if they had undetectable HCV RNA on completion of antiviral therapy. Patients who had detectable HCV RNA on treatment completion were defined as non-responders (NR). Serum was collected at the time of liver biopsy following an overnight fast for 8 hours. Standard biochemical tests were performed using a Hitachi 747-100 Analyser (Roche, Australia). Circulating insulin and c-peptide levels were determined using the Tosoh AIA600 analyzer, 2-site immunoenzymometric assays (Tosoh Medics, San Francisco, USA). Insulin resistance was determined using the homeostasis model of assessment (HOMA).[29]
Real-time (RT)-PCR Methods
The steady state mRNA levels of PEPCK, TNF-α, SOCS-3 and IFN-R1 were assessed by semi-quantitative RT-PCR assays, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Total RNA was extracted from liver biopsy tissue (n=73) according to the Trizol method (Invitrogen, Melbourne, Victoria, Australia). cDNA was prepared by reverse transcription as previously described.[30]

The probe and primer sequences for GAPDH and TNF-α were as described previously.[30] Primer sequences for IFN-R1, SOCS-3, and PEPCK were designed using online software Primer 3, (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3www.cgi) (Table 1) and were purchased from Proligo Australia Pty Ltd (Lismore, New South Wales, Australia). PEPCK and TNF-α mRNA levels were determined by RT-PCR using a TAMRA-FAM probe as previously described.[30] mRNA expression of SOCS-3 and IFN-R1 was determined using SYBR green chemistry. Five µL of diluted (1/20) cDNA was added to a PCR mix containing 6.70µL sterile water, 12.5µL 2x SYBR mix (Qiagen, Clifton Hill, Victoria, Australia), 0.4µL each of forward and reverse primers to make up a final volume of 25µL. The cycling conditions for amplification were 95°C for 15 minutes, followed by 40 cycles of 94°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds in a Rotorgene 3000 (Corbett Robotics, Brisbane, Australia). Each assay was performed in duplicate and the analysis was performed using Rotorgene Analysis Software (Corbett Robotics, Brisbane, Australia).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOCS-3</td>
<td>5'-CCCTGACCCACCTACTGAA-3'</td>
<td>5'-TCCGACAGAGATGCTGAAGA-3'</td>
<td>NA</td>
</tr>
<tr>
<td>IFN-R1</td>
<td>5'-GTGGAACAGGAGGCCAGTGAAT-3'</td>
<td>5'-CAACCTCTACCCCATGAAGAAGTG-3'</td>
<td>NA</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-CCCCAGGGACCTCTCTCTTAA-3'</td>
<td>5'-CAGCTTGAGGTTTGCTACA-3'</td>
<td>5'-AGGAGGACCCAGGCAAT-3'</td>
</tr>
<tr>
<td>PEPCK</td>
<td>5'-AGCTGGCAAACATGGAGTCTT-T-3'</td>
<td>5'-CCTCGGAACCAGTTGACAT-3'</td>
<td>5'-CGCTTCTTTGGCTACAAGTTCGGCA-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-TGCAACCAACTGCTTAGC-3</td>
<td>5'-GGCATGGACTGTGGCATGAG-3'</td>
<td>5'-CCCTTCTCGGAAGTCAACTT-3'</td>
</tr>
</tbody>
</table>
The relative concentrations of mRNAs present were determined as previously described.[30] For each gene, the average of the duplicate assays was obtained and normalized to the average amount of GAPDH for each sample to determine relative changes in mRNA expression.

Immunohistochemistry for SOCS-3
Formalin-fixed paraffin-embedded liver biopsy samples from 38 of the subjects infected with HCV Genotype 1 or 4 who subsequently received antiviral therapy were used for immunohistochemical analysis of SOCS-3 expression as previously described. [30] The primary antibody used in the study was anti-SOCS-3 (Fusion Antibodies, Belfast, Northern Ireland, dilution 1/100). Tissue sections were photographed using a PixeLink Colour Digital Camera (Total Turnkey Solutions, Mona Vale New South Wales, Australia) mounted on an Olympus BX-40 microscope (Olympus Australia Pty Ltd, Mt Waverley, Victoria, Australia).

Image analysis was used to quantify the immunoreactivity of SOCS-3. A minimum of 30 non-overlapping fields at a magnification of X400 were photographed. Image analysis software (Image Pro Plus 4.5, SciTech Pty Ltd, Preston, Victoria) was used to assess the mean immunoreactive area per biopsy. The percent positive area was defined as the ratio of pixels set above the segmentation threshold to the total number of pixels within a defined area of interest. For each section, the percent positive area for replicate fields was averaged.

Statistical analysis
Continuous normally distributed variables were represented graphically as mean±standard error of the mean (SEM). Grade of steatosis, stage of fibrosis and alcohol consumption were summarized using the median. Chi square or Fisher’s exact tests were used to determine the differences in patient distribution between RES and NR and between SVR and NR for variables such as gender, cirrhosis on pre-treatment biopsy, the presence of steatosis and BMI categories. To compare the means of normally distributed variables, analysis of variance (ANOVA) or student’s t-tests were performed. To determine differences between groups for non-normally distributed variables, medians were compared using the Mann-Whitney U test. Pearson’s correlation coefficient was used to measure the degree of association between continuous normally distributed variables. The degree of association between non-normally distributed or ordinal variables was assessed using Spearman’s non-parametric correlation. Binary logistic regression was used to determine discrete factors associated with NR, adjusting for age at treatment, gender, previous alcohol consumption, the presence of steatosis or cirrhosis, type of treatment received, viral genotype and BMI. ANCOVA was performed to identify predictors of normally distributed variables such as SOCS-3, adjusting for factors such as age, gender, BMI, HOMA, cirrhosis on pre-treatment biopsy, previous alcohol consumption and the presence of steatosis. A backward elimination approach was used to remove non-significant variables and determine the most parsimonious model. All analysis was carried out using SPSS software version 11.0 (SPSS Inc. Chicago, IL, USA). Statistical significance was taken at the 95% confidence interval.
Results

Patient characteristics and antiviral regimes
Out of a total of 218 patients, 145 (95% Caucasian, 5% Asian) received antiviral therapy and 73 (100% Caucasian) comprised the RT-PCR cohort. Overall, the mean age of the patients was 41.2 ± 0.6 years and 156 (72%) were male. Viral genotype was 1 in 99 (45.4%), 2 in 5 (2.3%), 3 in 112 (51.3%) and 4 in 2 (1%). BMI was classified as lean in 101 patients (46.3%), overweight in 84 (38.5%) and obese in 33 (15.1%). Median prior alcohol intake was 20 (3-60) g/day. Thirty-three patients (15.1%) had cirrhosis on liver biopsy. One hundred (45.9%) patients had no steatosis, 71 (32.6%) had mild steatosis and 47 (21.5%) had moderate or severe steatosis. The mean fasting insulin level (available for 146 patients) was 9.1 ± 0.64 mU/L, the mean HOMA score was 2.02 ± 0.19 and mean c-peptide level was 0.73 ± 0.03 nmol/L. Within each viral genotype, there were no significant differences between patients who received antiviral therapy and those in the RT-PCR group except that those who received therapy were slightly older (42.3 ± 0.7 and 39.0 ± 1.0 years respectively, p=0.018).

A total of 145 subjects fulfilled the criteria for the treatment arm of the study; 128 patients received combination therapy with ribavirin and either standard IFN (n=108) or PEG-IFN (n=20) and 17 patients received IFN (n=14) or PEG-IFN (n=3) monotherapy.

Effect of host factors on treatment outcome
NR was seen in 36 patients (55%) with HCV genotypes 1 or 4 and 17 (22%) with viral genotypes 2 or 3 (Table 2). Among patients with viral genotype 1 or 4, subjects with NR were more likely to have cirrhosis on pre-treatment biopsy (p=0.003) and to be obese (p=0.004), however there was no difference in the prevalence or severity of steatosis or mean levels of circulating insulin, c-peptide and HOMA scores between patients with NR and RES. In patients with viral genotypes 2 or 3 there was no significant difference in host characteristics between subjects with NR or those with RES (Table 2). Median alcohol intake did not differ between subjects with NR or RES (data not shown).
Table 2: Clinical and demographic characteristics of responders and non-responders to antiviral therapy.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Genotypes 1 and 4 (n=66)</th>
<th>Genotypes 2 and 3 (n=79)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RES (n=30)</td>
<td>NR (n=36)</td>
</tr>
<tr>
<td>Number of patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>20 (67%)</td>
<td>31 (86%)</td>
</tr>
<tr>
<td>Age</td>
<td>42.2 ± 1.3</td>
<td>43.7 ± 1.0</td>
</tr>
<tr>
<td>Pre-treatment biopsy:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>2 (7%)</td>
<td>14 (39%)</td>
</tr>
<tr>
<td>Steatosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>17 (57%)</td>
<td>15 (42%)</td>
</tr>
<tr>
<td>Mild</td>
<td>10 (33%)</td>
<td>15 (42%)</td>
</tr>
<tr>
<td>Moderate/Severe</td>
<td>3 (10%)</td>
<td>6 (17%)</td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-obese</td>
<td>29 (97%)</td>
<td>25 (69%)</td>
</tr>
<tr>
<td>Obese</td>
<td>1 (3%)</td>
<td>11 (31%)</td>
</tr>
<tr>
<td>Fasting insulin (mU/L)</td>
<td>9.71 ± 2.47</td>
<td>9.98 ± 1.50</td>
</tr>
<tr>
<td>HOMA (mU/L)</td>
<td>1.95 ± 0.045</td>
<td>2.31 ± 0.48</td>
</tr>
<tr>
<td>C-peptide (nmol/L)</td>
<td>0.77 ± 1.79</td>
<td>0.86 ± 0.95</td>
</tr>
</tbody>
</table>

*) Fisher’s Exact test; †Mann-Whitney t-test; ‡Chi-squared test. Data are represented as mean±SEM.

Following multivariate analysis, variables independently associated with NR were viral genotype 1/4 (p<0.001), the presence of cirrhosis on pre-treatment biopsy (p=0.025) and obesity (p=0.010) (Table 3). When comparing patients with NR to those with SVR, these factors remained independently associated with treatment non-response (data not shown).

Table 3: Factors independently associated with non-response to treatment.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>p value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral genotype 1 or 4</td>
<td>&lt;0.001</td>
<td>4.1 (1.9-8.9)</td>
</tr>
<tr>
<td>Cirrhosis on pretreatment biopsy</td>
<td>0.025</td>
<td>3.2 (1.2-9.0)</td>
</tr>
<tr>
<td>Obesity*</td>
<td>0.010</td>
<td>3.9 (1.4-11.2)</td>
</tr>
</tbody>
</table>

*Caucasian ≥30kg/m², Asian ≥25kg/m²

In comparison with non-obese patients, obese subjects with viral genotype 1/4 were more likely to have steatosis (p<0.001) and had higher fasting serum insulin (p=0.041) and c-peptide (p=0.041) levels and higher HOMA scores (0.026). In
patients with viral genotype 2/3, obesity was also associated with higher grades of steatosis ($p=0.035$). However there was no significant difference in fasting serum insulin and c-peptide levels and HOMA scores between obese patients and non-obese subjects (Table 4) for patients with genotype 2/3.

**Table 4:** Treatment response, histological data and metabolic risk factors in non-obese and obese patients with chronic HCV.

<table>
<thead>
<tr>
<th>Genotypes 1 and 4</th>
<th>Non-obese</th>
<th>Obese</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>54</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Response to treatment</td>
<td>RES 29 (54%)</td>
<td>1 (8%)</td>
<td>0.004*</td>
</tr>
<tr>
<td></td>
<td>NR 25 (46%)</td>
<td>11 (92%)</td>
<td></td>
</tr>
<tr>
<td>Cirrhosis on pre-treatment biopsy</td>
<td>10 (19%)</td>
<td>6 (50%)</td>
<td>0.024*</td>
</tr>
<tr>
<td>Steatosis</td>
<td>None 31 (57%)</td>
<td>1 (8%)</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td></td>
<td>Mild 20 (37%)</td>
<td>5 (42%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moderate/Severe 3 (6%)</td>
<td>6 (50%)</td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.74 ± 0.09</td>
<td>5.11 ± 0.46</td>
<td>0.46‡</td>
</tr>
<tr>
<td>Insulin (mU/L)</td>
<td>8.41 ± 1.41</td>
<td>14.63 ± 2.58</td>
<td>0.041‡</td>
</tr>
<tr>
<td>CPEP (nmol/L)</td>
<td>0.73 ± 0.10</td>
<td>1.14 ± 0.17</td>
<td>0.041‡</td>
</tr>
<tr>
<td>HOMA</td>
<td>1.75 ± 0.29</td>
<td>3.54 ± 1.05</td>
<td>0.026‡</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotypes 2 and 3</th>
<th>Non-obese</th>
<th>Obese</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>68</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Response to treatment</td>
<td>RES 55 (81%)</td>
<td>7 (64%)</td>
<td>0.24*</td>
</tr>
<tr>
<td></td>
<td>NR 13 (19%)</td>
<td>4 (36%)</td>
<td></td>
</tr>
<tr>
<td>Cirrhosis on pre-treatment biopsy</td>
<td>7 (13%)</td>
<td>1 (9%)</td>
<td>0.88*</td>
</tr>
<tr>
<td>Steatosis</td>
<td>None 26 (38%)</td>
<td>2 (18%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mild 25 (37%)</td>
<td>2 (18%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moderate/Severe 17 (25%)</td>
<td>7 (64%)</td>
<td>0.035†</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.78 ± 1.46</td>
<td>4.81 ± 0.35</td>
<td>0.95‡</td>
</tr>
<tr>
<td>Insulin (mU/L)</td>
<td>7.42 ± 0.85</td>
<td>12.99 ± 3.23</td>
<td>0.13‡</td>
</tr>
<tr>
<td>CPEP (nmol/L)</td>
<td>0.62 ± 0.41</td>
<td>0.95 ± 0.17</td>
<td>0.080‡</td>
</tr>
<tr>
<td>HOMA</td>
<td>1.60 ± 0.17</td>
<td>3.06 ± 0.97</td>
<td>0.18‡</td>
</tr>
</tbody>
</table>

*) Fischer’s Exact test; †) Chi-squared test; ‡) Mann-Whitney U-test. Data are represented as mean±SEM.

Eleven of the 12 obese subjects with genotype 1/4 and 9 of 11 obese subjects with genotype 2/3 had steatosis, illustrating the close inter-relationship between these factors. Twenty-three of 54 non-obese subjects (43%) with genotype 1 and 42 of 68 non-obese subjects (63%) with genotype 2/3 also had steatosis. In these non-obese subjects, the presence of steatosis did not impair the ability to achieve a RES (viral genotype 1/4, $p=0.50$; viral genotype 2/3, $p=0.21$).

**Relationship between obesity, hepatic insulin sensitivity and SOCS-3**
To determine the effect of obesity on factors that may impair the IFN signalling cascade, PEPCK, TNF-α, SOCS-3 and IFN-R1 mRNA levels were determined by RT-PCR in 73 patients with chronic HCV.

In patients infected with viral genotype 1 (n=35), obese subjects had increased hepatic expression of SOCS-3 mRNA compared to lean subjects (p=0.047). By univariate analysis, SOCS-3 mRNA was associated with body mass index (p=0.047), but with no other demographic or histologic feature. Following multivariate analysis adjusting for age, gender, presence of cirrhosis, steatosis and HOMA score, the relationship between SOCS-3 mRNA expression and obesity remained significant (p=0.023).

Similarly, obese subjects had increased hepatic expression of PEPCK mRNA compared to lean subjects (p<0.01) and this relationship remained significant after multivariate analysis adjusting for age, gender, presence of cirrhosis, steatosis and HOMA score (p<0.001). In lean subjects, there was a significant inverse relationship between HOMA score and hepatic PEPCK mRNA levels (r=-0.45, p=0.01), consistent with the role of insulin in regulation of PEPCK gene expression. In contrast, in overweight and obese subjects, there was no association between HOMA score and PEPCK mRNA levels (r=0.004, p=0.98). These results provide circumstantial evidence that in comparison with lean patients, our obese subjects had impaired hepatic insulin sensitivity. Although we did not find a relationship between SOCS-3 expression and markers of systemic insulin resistance such as HOMA, insulin and c-peptide, there was a significant association between SOCS-3 mRNA levels and PEPCK mRNA levels (r=0.5, p=0.0092). This may reflect a relationship between SOCS-3 expression and hepatic insulin resistance.

The increase in expression of TNF-α in obese subjects compared with lean subjects approached significance (p=0.071) (Figure 1). In patients with viral genotype 1, there was a significant correlation between TNF-α and PEPCK mRNA levels (r= 0.34, p<0.001) and between TNF-α and SOCS-3 mRNA levels (r=0.72, p<0.001).

In patients infected with viral genotype 3, there was no significant difference in SOCS-3, PEPCK and TNF-α mRNA expression between obese and lean subjects (p=0.46, p=0.67, p=0.59 respectively). No relationship was seen between IFN-R1 mRNA levels and BMI or other markers of insulin resistance for all genotypes (data not shown).

**Increased hepatic SOCS-3 expression in patients with treatment non-response.**

To address the association between SOCS-3 expression and response to antiviral therapy, immunohistochemistry for SOCS-3 was performed in liver sections from subjects infected with HCV Genotype 1 or 4 who subsequently received antiviral therapy. Immunoreactive product was seen in liver biopsies from all patients who were treatment non-responders, and all patients except for one, who were responders. Staining was present predominantly in hepatocytes and was localised to the cytoplasm. Hepatocytes were positive in periportal and other areas of the lobules. Immunoreactive staining was also present in bile ducts, but only infrequently in Kupffer cells and portal macrophages. (Figure 2)
Compared with responders, patients who were non-responders had significantly higher mean levels of SOCS-3 immunoreactivity (p=0.014) (Figure 3.). After correcting for age, gender, the presence of cirrhosis on pre-treatment biopsy, HOMA score, previous ethanol consumption, steatosis and BMI, increased SOCS-3 protein expression was independently associated with treatment NR in patients with viral genotype 1 (p<0.0001).

SOCS-3 immunoreactivity was significantly higher in non-lean subjects compared to lean subjects (p<0.013). By univariate analysis, SOCS-3 immunoreactivity was not associated with any other demographic, metabolic or histologic feature. Following multivariate analysis adjusting for age, gender, presence of cirrhosis, steatosis and HOMA score, the relationship between SOCS-3 immunoreactivity and obesity remained significant (p=0.012).

**Discussion**

In this single centre study, obesity was independently associated with non-response to antiviral therapy in subjects infected with HCV genotype 1. Virtually all obese subjects (87%) had steatosis, underlining the inter-relationship between these variables. However, steatosis was also present in 55% of non-obese subjects. In these latter patients, the presence or severity of hepatic steatosis did not influence the ability to achieve a response to treatment. In support of these findings, a recent study by Bressler et al, also demonstrated that obesity but not hepatic steatosis was an independent negative predictor of response to HCV treatment.[9]

Many previous studies of both standard and pegylated interferon have identified body weight as a factor impacting on treatment response rates.[11][12][13][14][31] It remains unclear whether weight-based dosing would improve the response rate or whether obesity is associated with a greater degree of resistance to antiviral therapy.[1] In this study we demonstrated that obese subjects infected with HCV genotype 1, had increased hepatic expression of SOCS-3, a factor that has been shown to inhibit IFN signalling. This relationship between obesity and increased SOCS-3 expression remained significant after correction for other factors associated with non-response to treatment.

Importantly, patients with HCV genotype 1 who were NR had significantly higher levels of SOCS-3 protein expression compared with RES. Engagement of IFN with its receptor activates receptor-associated tyrosine kinases that phosphorylate signal transducer and activator of transcription (STAT) factors 1 and 2.[32][33] Phosphorylated STAT proteins migrate to the nucleus and induce the transcription of several antiviral target genes. This IFN signalling pathway is down-regulated by members of the SOCS family of proteins.[34] [18] SOCS 1 and 3 have been shown to inhibit the tyrosine phosphorylation and nuclear translocation of STAT 1 in response to IFN stimulation and this inhibition occurs at very low levels of SOCS protein expression.[35]

Several factors including the HCV core protein [17], liver toxins [22] and various cytokines [36][37] have been shown to induce hepatic SOCS-3 expression. In chronic HCV, TNF-α may have a key role in this inhibitory pathway. TNF-α levels are increased in the serum, [38] liver and peripheral blood mononuclear cells [20] of subjects with chronic HCV compared with control subjects. Higher pre-treatment intrahepatic [19] and PBMC [20] TNF-α mRNA levels were observed in patients who
subsequently failed to respond to IFN therapy compared with those subjects who had an SVR. More recently, Hong and colleagues have shown in a mouse model, that injection of TNF-α markedly induced expression of SOCS-3, resulting in inhibition of IFN signalling in hepatic cells.[22] In our cohort of patients with chronic HCV there was a striking correlation between TNF-α and SOCS-3 mRNA levels, consistent with a role for this cytokine in induction of SOCS-3 expression in vivo.

Importantly, the expression of SOCS-3 in the liver is induced by cytokines and hormones that are associated with obesity-dependent insulin resistance.[39] Up-regulation of hepatic SOCS-1 and-3 mRNA and proteins have been observed in various insulin-resistant animal models.[26] A marked decrease in SOCS-3 expression was found in obese mice lacking TNF-α signalling, supporting the premise that the elevated levels of SOCS-3 in obesity may be related to increased TNF-α expression.[40] In our study, obese subjects with viral genotype 1 had increased hepatic expression of PEPCK compared with non-obese subjects, consistent with impaired hepatocyte insulin sensitivity. PEPCK catalyses a key step in gluconeogenesis and insulin decreases the transcription of its gene.[41] Insulin resistance is associated with a failure of insulin to suppress the activity of enzymes involved in gluconeogenesis leading to increased hepatic glucose production.[42] In our cohort of subjects with chronic HCV, highly significant correlations were seen between hepatic PEPCK and both TNF-α and SOCS-3 mRNA levels. Similar to their inhibitory role in IFN signalling, TNF-α and SOCS-3 may act as negative regulators in insulin signalling.[25]

In our patients infected with viral genotype 3, there was no statistically significant difference in markers of insulin resistance or hepatic gene expression of PEPCK, SOCS-3 and TNF-α between obese and non-obese patients. It remains unclear why the effect of obesity on hepatic IR and expression of SOCS-3 was seen in subjects with viral genotype 1 but not genotype 3. The prevalence of cirrhosis was higher in the cohort of subjects infected with viral genotypes 1 or 4, and this may have contributed to an overall increase in insulin resistance in this population. However the relationships between obesity and expression of PEPCK, SOCS-3 or TNF-α were found to be independent of the presence of cirrhosis. Interestingly, a previous study has also shown that subjects with genotype 3 have lower levels of insulin resistance compared with other viral genotypes.[43] In addition, the increased risk for the development of type 2 diabetes in chronic HCV appears to be largely among non-genotype-3 infected subjects.[43][44][45] In contrast to the effect of obesity on hepatic gene expression, no differences in mRNA levels studied were seen between subjects with or without hepatic steatosis.

A number of earlier studies have demonstrated that a sustained response to antiviral therapy is dependent on high levels of expression of the IFN receptor.[46][47] Information regarding the regulation of expression of the interferon receptor in chronic HCV remains limited. In our study, we found no difference in the expression of the IFN-R1 between obese and non-obese subjects. Although a previous study demonstrated a down-regulation of IFN-R1 mRNA in fibrotic livers [48], we did not observe this finding in our subjects with chronic HCV.

In summary, in our cohort of patients with chronic HCV, BMI ≥ 30kg/m² was independently associated with non-response to antiviral therapy. In subjects with viral
genotype 1, obesity was associated with increased PEPCK mRNA levels consistent with impaired hepatocyte insulin sensitivity and with increased expression of TNF-α and SOCS-3, factors that may reduce interferon signalling. Induction of hepatic SOCS-3 expression may be one mechanism by which obesity reduces the biologic response to IFN in patients infected with viral genotype 1.

Conflict of Interest Statement
The authors have no conflict of interest in relation to the publication of this manuscript.
References


Legends to Figures

Figure 1: (A) PEPCK, (B) SOCS-3 and (C) TNF-α mRNA levels in lean (BMI < 25 kg/m²), overweight (BMI 25-29.9 kg/m²) and obese patients (BMI ≥ 30 kg/m²) with chronic HCV Genotype 1. Data are presented as mean ± SEM. *p<0.05 obese compared with lean patients **p<0.01 obese compared with lean patients

Figure 2: Immunohistochemical analysis of SOCS-3 protein expression in patients with chronic hepatitis C. (A) Obese treatment non-responder. Strong staining is present predominantly in hepatocytes and is localised to the cytoplasm. (B) Treatment responder. (C) Negative control (A, B & C - original magnification X400)

Figure 3: SOCS-3 protein immunoreactivity (percentage area positive) in liver sections from patients infected with chronic HCV viral genotype 1/4 who were non-responders (NR) or responders (RES) to antiviral treatment. Data are represented as mean ± SEM. *p<0.05
Figure 1.

**A**  Relative PEPCK mRNA Expression

**B**  Relative SOCS-3 mRNA Expression

**C**  Relative TNF-α mRNA Expression
Figure 2
SOCS-3 Protein Expression (% area positive)

Figure 3

RES
NR

*
Non-response to antiviral therapy is associated with obesity and increased hepatic expression of SOCS-3 in patients with chronic hepatitis C, viral genotype 1

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